Enzyme Immunoassay for
Active Rat tPA

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INTRODUCTION

Tissue-type Plasminogen Activator (tPA) is a member of the serine proteinase family. tPA functions to lyse fibrin clots into soluble plasmin fragments. tPA is active in two forms, single-chain and two-chain. The two-chain tPA is created via interaction with the plasmin product cleaving the single chain. This two-chain form is regarded as the more active form.

Both single-chain and two-chain tPA are complexable with PAI-1. PAI-1 acts as an inhibitor for tPA by binding to the tPA and thus stifling its ability to lyse fibrin.

tPA can serve as an indicator of both myocardial infarction for patients with impaired fibrinolytic systems as well as a marker for type-II diabetes.

PRINCIPLES OF PROCEDURE

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of active tPA levels in biological fluid. This test kit operates on the basis of sandwich ELISA where free, active, tPA enzyme complexes with PAI-1 and is quantified with the use of an HRP labeled secondary antibody.

First the biotinylated PAI-1 binds to the avidin coated wells. Next, active tPA present in the standard or unknown, complexes with PAI-1. Inactive or complexed tPA is removed in a subsequent wash step. A primary antibody specific for tPA is then added to each well followed by the HRP conjugated secondary antibody. The bound conjugated secondary antibody is detected by the addition of substrate, which generates an optimal color after 10 minutes. Quantitative test results may be obtained by the measure and comparison of the sample and standard absorbance readings when read with a microplate reader at 450 nm.

MATERIALS PROVIDED

Component	Contents	Quantity	Storage
Coated Plate	Coated 96-well plate	1 plate	4°C
Rat tPA Activity Standard	Lyophilized active rat tPA standard	1 vial	4°C
Biotinylated PAI-1	Lyophilized biotinylated PAI-1	1 vial	4°C
10x TBS Buffer	10x Buffer for acidic samples	5 mL	4°C
10x Wash Buffer	10x Solution for washing plate	50 mL	4°C
Substrate	TMB substrate	10 mL	4°C
Primary Antibody	Lyophilized anti-mouse tPA antibody	1 vial	4°C
Secondary Antibody	HRP-conjugated secondary antibody	1 vial	4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Adjustable pipettes (10-1,000 µL) and disposable tips
2. Beakers, flasks, and cylinders as necessary for preparation of reagents
3. Microplate reader with 450 nm filter
4. Deionized Water
5. 1 N Sulfuric Acid
6. TBS Buffer (see Reagent Preparation)
7. Blocking Buffer (see Reagent Preparation)
8. Microplate shaker with uniform horizontal circular movement up to 300 rpm
9. Plastic film or plate cover to cover plate during incubation

STORAGE CONDITIONS

1. Store this kit and its components at 4°C until use.
2. Do not freeze.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions, or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
3. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

PROCEDURAL NOTES

1. This assay should be performed at room temperature.
2. Performance of the entire kit at once is not required. When performing this kit in part, please adhere to the following:

   - All unused components should be returned to storage at 4°C.

   - Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Seal with a heat sealer. If a heat sealer is not available, thoroughly close the open end with tape. Try to remove excess air before sealing.

   - If not using the entire plate at once, then prepare only the required amount of reagents where appropriate.

   - tPA Standard and reconstituted biotinylated PAI-1 stock solutions must be stored at –70 °C after initial use.
3. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

SAMPLE COLLECTION AND PREPARATION

Samples should be collected using trisodium citrate, acidified citrate or Stabilite™ (DiaPharma) collection media. Collection should be in accordance with the collection vials manufacturers instructions or in a 1:10 ratio of collection media to blood.

Immediately, upon collection of blood, the samples should be centrifuged at 3000 x g. This should ensure the removal of platelets as they can release PAI-1 that in turn complexes with uPA. The plasma can be transferred to a clean plastic tube and stored frozen for up to one month. Samples are stable for

approximately 5 hours when stored at 4° C with the Sabilyte[tm] media or up to one month when stored at -20°C.

Note: Detergents such as Triton X cause interference with the assay. If using detergent extracted samples, it is necessary to dialyze the samples overnight to remove the detergent.

REAGENT PREPARATION

The following solutions should be prepared fresh before starting the assay.

1. **TBS Buffer**: Dilute the 10x TBS Buffer to 1x with deionized water and mix prior to use.
2. **Blocking Buffer**: 3% BSA in TBS Buffer.
3. **Wash Buffer**: Dilute the 10x Wash Buffer to 1x with deionized water and mix prior to use.

ASSAY PROCEDURE

1. Remove microplate from the bag.
2. Make a working solution of Biotinylated PAI-1 by adding 10 mL of 3% BSA Blocking Buffer directly to the vial and mix gently to dissolve the contents.
3. Add 100 µL of the Biotinylated PAI-1 Solution to both the standard and test wells.
4. Shake the plate at 300 rpm on a plate shaker for 30 minutes.
5. Wash wells according to the following wash procedure:
   a. Remove contents of the plate by inversion into an appropriate disposal device.
   b. Tap out remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of Wash Buffer.
   d. Let stand for 2-3 minutes.
   e. Repeat procedure 2 more times and then proceed to step "f".
   f. Remove contents of the plate by inversion into an appropriate disposal device.
   g. Tap out the remaining contents of the plate onto a lint free paper towel then proceed to step 6.

   Note: The decanted wells should be void of visible moisture before proceeding. If moisture is still visible then follow step "g" until satisfactory results are obtained.
6. Reconstitute the standard by adding 1.0 mL of 3% Blocking Buffer to the standard vial. This stock solution is now 1,000 ng/mL. Prepare the Standards as indicated in the table below.

   Note: The standards should be applied to the plate immediately upon preparation.

Standard	tPA Concentration (ng/mL)	Amount of 3% BSA (µL)	Transfer Volume (µL)	Transfer Source	Final Volume (µL)
S_{10}	50	950	50	Stock	500
S_9	25	500	500	S_{10}	600
S_8	10	600	400	S_9	500
S_7	5	500	500	S_8	600
S_6	2	600	400	S_7	500
S_5	1	500	500	S_6	500
S_4	0.5	500	500	S_5	500
S_3	0.25	500	500	S_4	600
S_2	0.1	600	400	S_3	500
S_1	0.05	500	500	S_2	1000
S_0	0	500	---	---	500

7. Add 100 µL of Standards or unknowns to the plate. See **Scheme I** for suggested template design.
8. Shake plate at 300 rpm on the plate shaker for 30 minutes.
9. Wash wells according to step 5 located above in this section.
10. Make a working solution of Primary Antibody by reconstituting the contents of the vial with 10 mL of 3% BSA Blocking Buffer.
11. Add 100 µL of the Primary Antibody Solution to each well.
12. Shake plate at 300 rpm on the plate shaker for 30 minutes.
13. Wash wells according to step 5 located above in this section.
14. Make a working solution of Secondary Antibody by reconstituting the contents of the vial with 10 mL of 3% BSA Blocking Buffer.
15. Add 100 µL of the Secondary Antibody Solution to each well.
16. Shake plate at 300 rpm on the plate shaker for 30 minutes.
17. Wash wells according to step 5 located above in this section.
18. Add 100 µL of TMB Substrate to each well and incubate for 2-10 minutes.
19. Stop the reaction with 50 µL per well of 1 N H_2SO_4 and read plate at 450 nm.
20. If accounting for substrate background, use two wells as blanks with only substrate in the wells (100 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

    NOTE: Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.

Scheme I: Suggested Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_{10}	S_9	S_8	S_7	S_6	S_5	S_4	S_3	S_2	S_1	B_0	U_1
B	S_{10}	S_9	S_8	S_7	S_6	S_5	S_4	S_3	S_2	S_1	B_0	U_1
C	U_2	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}
D	U_2	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}
E	U_{14}	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}
F	U_{14}	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}
G	U_{26}	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}
H	U_{26}	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}

CALCULATIONS

1. Subtract the average O.D. value of the blank wells (BLK) from all other pairs of wells.
2. Average the O.D. values for each pair of duplicate wells.
3. Plot a standard curve using the average O.D. value for each standard value versus the concentration of standard.
4. Determine the concentration of each unknown by interpolation from the standard curve.

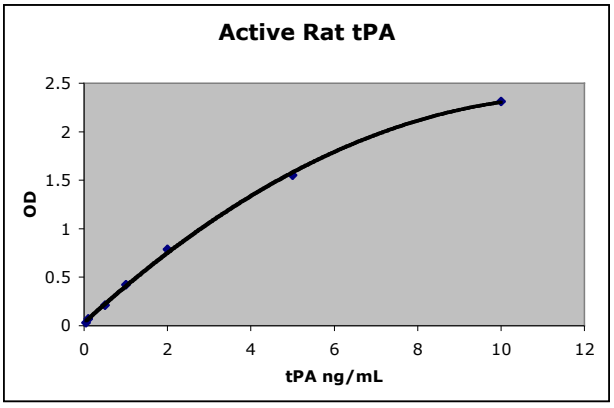
TYPICAL RESULTS

Note: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life or due to lot variance.

Typical Data:

Standard	[tPA] (ng/mL)	O. D.
S_1	0.05	0.030
S_2	0.1	0.030
S_3	0.25	0.071
S_4	0.5	0.212
S_5	1.0	0.423
S_6	2.0	0.789
S_7	5.0	1.553
S_8	10.0	2.310

Figure 1: Typical Standard Curve



PERFORMANCE CHARACTERISTICS

Assay Range: 0.05- 10 ng/mL

Samples with tPA levels higher than 10 ng/mL should be diluted in similar media devoid of active tPA.

REFERENCES

1. Thogersen A et al. (1998) Circulation 98: 2241-2247
2. Eliasson M et al. (2003) Cardiovascular Diabetology 2:19

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